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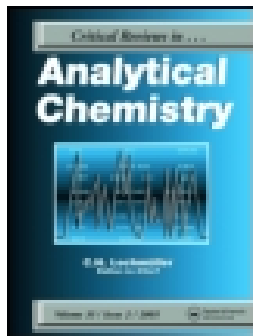
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The Measurement of Ammonia in Human Breath and its Potential in Clinical Diagnostics

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Abstract

Ammonia is an important component of metabolism and is involved in many physiological processes. During normal physiology, levels of blood ammonia are between 11 and 50 μM . Elevated blood ammonia levels are associated with a variety of pathological conditions such as liver and kidney dysfunction, Reye's syndrome and a variety of inborn errors of metabolism including urea cycle disorders, organic acidaemias and hyperinsulinism/hyperammonaemia syndrome in which ammonia may reach levels in excess of 1 mM. It is highly neurotoxic and so effective measurement is critical for assessing and monitoring disease severity and treatment. Ammonia is also a potential biomarker in exercise physiology and studies of drug metabolism. Current ammonia testing is based on blood sampling, which is inconvenient and can be subject to significant analytical errors due to the quality of the sample draw, its handling and preparation for analysis. Blood ammonia is in gaseous equilibrium with the lungs. Recent research has demonstrated the potential use of breath ammonia as a non-invasive means of measuring systemic ammonia. This requires measurement of ammonia in real breath samples with

associated temperature, humidity and gas characteristics at concentrations between 50 and several thousand parts per billion. This review explores the diagnostic applications of ammonia measurement and the impact that the move from blood to breath analysis could have on how these processes and diseases are studied and managed.

Introduction

Ammonia is an important analyte in clinical diagnostics. It is an inorganic nitrogen compound found naturally in the body and is involved in many metabolic processes. The clinical standard for measuring ammonia is through blood analysis. However, due to the challenges and complications associated with blood ammonia sampling, there has been a significant increase in interest in other sample matrices in which to measure ammonia (Metz, 2014). In particular, there has been major development in the field of breath ammonia measurement (Hibbard and Killard, 2011). Exhaled human breath typically consists of nitrogen (78.6%), oxygen (16%), carbon dioxide (4.5%), inert gases and volatile organic compounds (VOCs) (0.9%) (Tortora, 2006). VOC levels in breath may vary between individuals (Gouma, 2012). VOCs are generated in the body as part of biochemical reactions and metabolic processes, but may also be inhaled, ingested or produced exogenously, for example by gut or oral bacteria. When absorbed into the blood stream, VOCs may cross the alveolar interface and appear in the breath exhalate in parts per million (ppm) to parts per trillion (ppt) concentrations (Smith and Spanel, 2007). These VOCs can be used to study human metabolism, determine health status, and diagnose disease (Lourenco and Turner, 2014). In spite of the potential advantages of breath VOCs and the increased research in this field in the last 20 years, few have yet been effectively applied to clinical diagnostics (Risby and Solga, 2006). Now, over two thousand compounds have been identified in breath and approximately 35 of these have been established as biomarkers (Wang and Sahay, 2009). Ammonia also has the potential to yield diagnostic and metabolic information (Smith, 2011). The physiological range for breath ammonia is 50 to several thousand ppbv (Kearney et al., 2002), and so only techniques capable of quantification in this range are relevant.

To date, a number of techniques have been developed to measure ammonia in breath at physiologically relevant concentrations in real (humidified) breath samples. These have mostly been instrumental techniques including chemical ionisation, gas chromatography-mass spectrometry (GC-MS) (Davies et al., 1997; Van den Velde et al., 2008), selected ion flow tube mass spectrometry (SIFT-MS) (Davies et al., 1997; Enderby et al., 2009; Turner et al., 2006), laser spectroscopy and laser photoacoustic spectroscopy (LPAS) (Hibbard and Killard, 2011; Lewicki et al., 2011; Navas et al., 2012; Popa et al., 2011; Wang et al., 2011). However, most of the developed instrumental techniques are typically complex, expensive and not suitable for diagnostic application. In addition, a number of the methods contribute to pre-analytical errors due to issues such as sample transportation and handling. While a number of sensor-based approaches have been developed including quartz crystal microbalance (Becker and Cooper, 2011; Ishida et al., 2008; Ogimoto et al., 2015), chemical and optical sensors, many of these have had challenges in terms of detection limits, or operation in real humidified breath samples, which has made them unsuitable for physiological application. However, some have now demonstrated effective application in human clinical studies (Hibbard et al., 2013). Several reviews are available on ammonia measurement techniques (Davies et al., 2014; Hibbard and Killard, 2011; Spanel and Smith, 2011). However, this is not the focus of this review.

This review focuses on the potential of breath ammonia monitoring and its application in clinical diagnostics. An overview of ammonia metabolism in the body and its key organs and systems is described. External factors that can influence ammonia metabolism are also highlighted. The diseases and conditions which are associated with systemic ammonia are also explored, along

with a description of the potential of breath ammonia measurement to be used to maintain health, diagnose and manage disease and monitor therapy.

The relationship between blood and breath ammonia

Blood ammonia is in the form of either ammonium ions ($\text{NH}_4^+_{(\text{aq})}$) or dissolved gaseous ammonia ($\text{NH}_{3(\text{g})}$). Throughout this review, unless stated otherwise, we will refer to these interchangeably as ammonia. Ammonia has a pKa of 9.3 and so under normal physiological conditions (pH 7.4) above 98% of ammonia is present as $\text{NH}_4^+_{(\text{aq})}$, as determined by the Henderson-Hasselbach equation (Solga et al., 2013):

Blood ammonia is the clinical standard for determination of systemic ammonia levels. While arterial blood ammonia is accepted as being most representative of systemic ammonia levels, sampling arterial blood is challenging. Venous ammonia varies somewhat from arterial ammonia, but is routinely used (Adeva et al., 2012). A major limitation of blood ammonia measurement is the complexity involved in the correct drawing and handling of the sample. Ammonia levels may spontaneously increase in a blood sample (Maranda et al., 2007). Difficult venepuncture, haemolysis of red blood cells (RBCs) and changes in metabolism can cause a sudden rise in the ammonia levels of the blood sample. Factors such as anxiety, exercise, smoking and alcohol intake may also affect systemic ammonia levels. Blood sampling remains a significant challenge, particularly in children and those with developmental impairment.

Breath analytics is based on the hypothesis that the concentrations of VOCs in blood can be related to their concentration in breath as humidified VOCs (Ogimoto et al., 2015). There are limitations to this hypothesis as VOCs can be produced in the oral cavity, airways, by bacteria in

the gut, or be emitted from mucus, saliva, and aerosols in the respiratory tract (Lourenco and Turner, 2014). It has been demonstrated that injecting dogs with ammonium acetate after elevating blood pH with NaHCO_3 to increase the level of gaseous ammonia led to an increase in expired ammonia (Robin et al., 1959). It is accepted that ammonia traverses the alveolar membrane through simple diffusion. Measurements of breath and blood ammonia, in which the expired air was allowed to bypass the upper airway were found to be in good agreement (Larson et al., 1979). A number of other studies have also examined the relationship between blood and breath ammonia concentrations. A colorimetric detection method with a limit of detection of 1 ppm was used to compare levels in chronic liver disease patients (Wakabayashi et al., 1997). While the results did show some relationship between blood and breath ammonia, the technique was not sensitive enough to accurately measure across the clinical range. A further study also demonstrated a slightly improved correlative relationship in chronic liver disease patients (Shimamoto et al., 2000). Again, however, the methodology which employed Tedlar bags and an ammonia electrode was not sufficiently sensitive to achieve a more definitive correlation. In addition, a correlation was not established between breath ammonia and psychometric testing or arterial blood ammonia in hepatic encephalopathy (HE) patients using an ammonia sensor based on a fiber optic with pH sensitive dye, which again had not been validated to be analytically rigorous at clinical concentrations, or been shown to be capable of dealing with the complexities of breath sample analysis (DuBois et al., 2005).

While few direct, and no definitive studies have yet shown the relationship between blood and breath ammonia, the existence of a relationship has been explored through the inferred relationship between hepatic dysfunction (i.e., liver disease) and associated HE, particularly in

studies investigating the breath ammonia concentrations in chronic liver disease and cirrhotic patients who are known to have elevated blood ammonia. However, many of these studies have also suffered from methodological flaws associated with the quality of the measurement system. Adrover et al. (2012) found little difference in the breath ammonia levels between healthy subjects and cirrhotic patients using an electrochemical measurement technique; a technique which was again untested and unvalidated in breath ammonia samples (Adrover et al., 2012). Dubois et al. achieved a weak correlation ($r = 0.31$, $p = 0.03$) between arterial ammonia and the number connection test for HE and negative correlation between breath ammonia level and number connection testing ($r = -0.55$, $p = 0.03$). No correlation was found between breath and arterial ammonia levels (DuBois et al., 2005). As a consequence, the link between breath ammonia, blood ammonia and hepatic disease remains to be adequately validated. Frustratingly, despite the power of techniques such as SIFT-MS and laser spectroscopy to be able to precisely and quantitatively measure ammonia at ppbv concentrations, no published studies have yet been performed with these systems to evaluate the correlation between blood and breath ammonia, and so the link between breath and blood ammonia levels - either direct or inferred -- while being highly probable, remains largely unproven.

Ammonia metabolism and disease

The importance of ammonia in metabolism has been known for a very long time (Dawson, 1978). It has been linked with numerous pathological conditions (Adeva et al., 2012). Under normal conditions, ammonia-rich blood is transported via the portal vein to the liver which goes on to be detoxified via the urea cycle and excreted by the kidneys as urea in urine, thus

maintaining nitrogen homeostasis (figure 1). Blood ammonia levels are typically in the range of 11-50 μM . Levels also vary between venous, capillary and arterial blood (Mehmood et al., 2013; Ong et al., 2003). Elevated blood ammonia can be caused by liver failure or impairment, some urinary tract infections, gastrointestinal bacterial overgrowth, Reye's syndrome, inherited defects of the urea cycle, and other metabolic disorders. These disorders and their associated ammonia levels are listed in table 1. When ammonia homeostasis is affected, there can be an increase in systemic ammonia (hyperammonaemia). Hyperammonaemia is not itself a diagnosis but a prompt for further investigation to find the underlying cause which may be inherited or acquired (Elgouhari and O'Shea, 2009). Ammonia toxicity can affect all organs, especially the brain. Levels exceeding 100 μM may trigger a cascade of pathological events in which the liver, kidney, stomach, lung, brain and central nervous system (CNS) may be irreversibly affected, leading to encephalopathy with associated neurological and cognitive impairment across a broad spectrum of severity (Bosoi and Rose, 2009; Pita et al., 2004).

Inherited hyperammonaemia is a neonatal emergency. Ammonia levels should be measured in seemingly healthy neonates with unexplained non-specific systemic illness with neurological symptoms (Leonard and Morris, 2006).

There is often no definitive cure for most hyperammonaemic conditions. However, restriction of dietary protein intake and prevention of catabolism through high calorie diets and supplements can prevent encephalopathy. Current treatment of hyperammonaemia involves lowering ammonia levels immediately by treating any ammonia producing processes which impacts on encephalopathy (stopping gastrointestinal bleeding, treating infections, kidney failure, and

electrolyte abnormalities). Management of ammonia levels then takes place by inhibiting ammonia production in the gut and targeting ammonia removal pathways. Management requires tailored intake of protein and a provision of carbohydrates (to stop catabolism and promote anabolism). Ammonia scavenging drugs (sodium benzoate, sodium phenylbutyrate and arginine hydrochloride) may also be used in some cases but it is uncommon as they are not all approved by regulatory agencies (Broomfield and Grunewald, 2012). Severe cases may require haemofiltration or dialysis. Medicines containing ammonium (including certain antacids) should also be avoided.

The role of the liver in ammonia metabolism

The liver maintains nitrogen homeostasis. It does this by converting nitrogenous compounds produced via the breakdown of amino acids into less toxic soluble forms which can be safely removed by the urea cycle (figure 2) and excreted by the kidneys (Adeva et al., 2012). The liver is subject to a number of inherited or acquired disorders, which can affect its ability to effectively metabolise ammonia to urea.

A urea cycle disorder (UCD) is a genetic mutation in one of the six enzymes that control the cycle: carbamoyl phosphate synthetase-1 (CPS-1), ornithine transcarbamylase (OTC), argininosuccinate synthetase-1, argininosuccinate lyase, arginase-1 hydrolysis (table 2). Protein breakdown results in an increase in glutamate concentration, which signals the up-regulation of N-acetylglutamate synthase (NAGS) and thus the entire cycle. The remaining enzymatic steps are controlled by their substrate concentrations. Impaired enzymatic function obstructs the cycle causing hyperammonaemia (Voet and Voet, 2004). Half of patients with UCD present in the

neonatal period with non-specific symptoms which develop into hyperammonaemic crisis and which results in severe intellectual disability (Leonard and Morris, 2002). Effective neonatal monitoring for elevated ammonia could reduce associated mortality and morbidity. Neonatal blood sampling is particularly problematic and so non-invasive breath testing could be extremely effective. Acquired UCD can present at any time. It is usually brought on by pregnancy, infectious illnesses or fasting with subsequent catabolism or the use of sodium valproate which unmasks latent CPS-1 or OTC deficiency (Gropman et al., 2007). Sodium valproate is known to cause hyperammonaemia (Aires et al., 2011). Diagnosis is performed by measuring ammonia levels and enzyme activity in leukocytes or cultured fibroblasts. Timely detection, close monitoring, diet and drug management are used to maintain blood ammonia at physiological levels (Daniotti et al., 2011). Patients with UCD and other chronic hyperammonaemic conditions have no effective means of monitoring their condition as blood ammonia self-testing is not effective. The use of a breath test could allow effective self-monitoring if adequate relationships between breath and blood ammonia levels can be established.

Cirrhosis is a scarring of the liver tissue as a result of long-term damage. This scarring cuts down on blood flowing through the liver, causing a loss of function. Patient sickness due to cirrhosis is measured with Child Pugh or Model for End-Stage Liver Disease (MELD) scores which are a measure of the risk of portosystemic shunting and redistribution of organ blood flow. The portal vein is a blood vessel from the gastrointestinal tract and spleen to the liver. In healthy individuals, ammonia levels in the portal vein are higher than in the hepatic vein because ammonia is removed by the liver. Patients with liver disease may develop portal collateral veins

(varices) that bypass the liver and divert portal blood with high ammonia levels to systemic circulation (figure 3) (Frontera, 2014; Imran et al., 2012; Luo et al., 2014).

Noiret et al. (2014) have developed a mathematical model of portosystemic shunting in cirrhosis to monitor hyperammonaemia to be used in conjunction with other monitoring techniques such as ammonia levels (Noiret et al., 2014). Ammonia has also been shown to drive dendritic immune cells into dysfunction which contributes to the immunocompromised state of cirrhosis (Auffermann-Gratzinger et al., 2001) and tumour patients (Luo et al., 2014).

Hepatic myelopathy (HM) is an unusual complication of chronic liver disease which manifests as cirrhosis and portosystemic shunts (Utku et al., 2005). It is characterised by spastic paraparesis which results in patients being confined to a wheelchair (Ben Amor et al., 2014). Ammonia has been identified as a major contributor to the development of HM (Campellone et al., 1996). Although ammonia-lowering treatment has not been shown to help, liver transplantation, along with Lioresal treatment has been shown to improve patient mobility (Campellone et al., 1996; Endre et al., 2011; Weissenborn et al., 2003). Reye's syndrome is characterised by cerebral oedema which often occurs during recovery from viral infection. It has also been linked to the use of aspirin. Changes occur to liver cells and so diagnosis is carried out via liver biopsy. Reye's syndrome also features hyperammonaemia of unclear cause and increased blood concentration of fatty and lactic acids (DeLong and Glick, 1982). Challenges remain with the management and monitoring of chronic liver disease, including the simplified measurement of ammonia levels.

Acute liver failure (ALF) is a rare but life threatening illness. It may rapidly lead to adverse events including systemic inflammatory response, renal failure, hyperammonaemia, cerebral oedema, HE, increased intracranial pressure (ICP), coma and death mainly due to ammonia toxicity (Cauli et al., 2014). While HE (Endre et al., 2011) and blood ammonia (Zhao et al., 2014) are used as markers for ALF, its diagnosis is quite complicated. It is important to note that encephalopathy can be delayed in some cases of ALF. Cirrhotic patients that present with neurological symptoms may be misdiagnosed with Parkinson's disease (Noone et al., 2008). Patients that present with high blood ammonia levels without liver disease may have an underlying liver issue such as cirrhosis or ALF which is often also associated with coagulopathy and hyperbilirubinaemia (Elgouhari and O'Shea, 2009). Liver disorders may also be asymptomatic until severe late stages of the disease and can develop into cancer. It is therefore imperative to monitor those with a history of hepatitis virus. Breath ammonia has been identified as a biomarker for liver disease (Adrover et al., 2012; DuBois et al., 2005; Shimamoto et al., 2000; Wakabayashi et al., 1997). Breath ammonia has the potential to diagnose, support and treat patients with underlying problems related to the liver such as those discussed above. A liver transplant can be considered when symptoms are life-threatening, although there may be severe complications. Future hopes for a definitive cure lie in gene replacement therapy (Gordon, 2003).

Most pharmaceutical drugs are metabolised by the liver and so most drugs have an impact on liver function. Many drugs are hepatotoxic, while others are used to treat liver dysfunction and associated hyperammonaemia. There are various ways to assess liver function using breath sampling, following the metabolism of various drugs (Armuzzi et al., 2002). In order to study ammonia-lowering drugs, oral glutamine is used to induce an increase in blood ammonia (Masini

et al., 2003). There are three ways to reduce ammonia levels; 1) decreasing ammonia synthesis (lactulose enema, BCAAs (Pencharz et al., 2012)), 2) inhibiting ammonia production (antibiotics, lactulose (Ait-Aissa and Aider, 2014), lactitol, modification of colonic flora with lactobacillus (Jiang and DuPont, 2005; So, 1992)), 3) ammonia removal (ornithine-aspartate, benzoate (Efrati et al., 2000)). Breath ammonia could also be used as a tool for studying the metabolism of other drugs for potential hepatotoxic effects so as to allow dosage to be optimised for individual patients; using a personalised or precision medicine approach (Finberg and Guharoy, 2012; Poh and Chang, 2012).

The role of the kidneys in nitrogen metabolism

Along with the liver, the kidneys also play an important role in nitrogen homeostasis (Weiner et al., 2014). Urea is passed into the blood stream by the liver and is absorbed by the kidneys via the glomerulus. The kidneys filter the blood urea. Excess ammonia is divided between the ureter for excretion as urine and the renal vein to be used in cellular metabolism. The kidneys play an important role in correcting acidosis by enhanced production or excretion of ammonia. A large acid load initiates ammonia excretion while a basic load initiates ammonia production (Garibotto et al., 2004). Human rhesus (Rh) proteins expressed in erythroid cells and epithelial tissues have been described as ammonia transporters. Rh-associated glycoprotein (RhAG) facilitates the movement of nitrogen compounds across RBC membranes. This process may contribute to the regulation of the systemic acid--base balance of the body (Ripoche et al., 2004). Metabolic acidosis is associated with chronic kidney disease as ammonia excretion decreases with declining filtration rate. Chronic kidney disease is progressive and results in end stage renal disease

(ESRD). It is treated with dialysis or can be cured through transplantation. Along with ESRD, many disorders can develop such as uraemia; acidosis/alkalosis and oedema. Creatinine, blood urea nitrogen (BUN) and glomerular filtration rate are important indicators of kidney function.

Currently, those with ESRD are managed using dialysis. Principally, haemodialysis is used, which is typically performed in a hospital or clinic. However, home dialysis and continuous ambulatory peritoneal dialysis are becoming increasingly used (Castledine et al., 2013). Breath ammonia has been used to study the efficacy of haemodialysis in end stage renal failure. Several studies on the efficacy of haemodialysis have shown good correlations between BUN and breath ammonia (Gouma et al., 2010; Hibbard et al., 2013; Narasimhan et al., 2001; Neri et al., 2012). Uraemia is an excess of creatinine and urea in systemic circulation. Breath ammonia levels have been used to study uraemia breath during haemodialysis, which has also been found to correlate with BUN (Mochalski et al., 2014; Romero-Gomez et al., 2001). However, anomalies still remain over the use of breath ammonia as a surrogate for BUN, as some have shown that there is no correlation between blood ammonia and blood nitrogen levels (Imran et al., 2012). The contribution of oral and/or alveolar (Hewitt and Nicholas, 1983) sources of ammonia generation is an area that needs to be studied more extensively to fully understand the interaction with blood nitrogen and ammonia in blood and breath (Cauli et al., 2014). Ammonia, monomethylamine, dimethylamine and trimethylamine are compounds known to also be present in kidney disease (Endre et al., 2011; Kohl et al., 2013; Narasimhan et al., 2001). Breath ammonia may prove useful, either alone or in combination with other compounds to assess kidney function. Treatment for kidney damage may include blocking N-methyl-D-aspartate (NMDA) receptors which delay kidney damage by allowing transient glomerulus filtration and ammonia elimination.

in order to delay hyperammonaemia. This treatment reduces changes in cerebral blood flow and brain lactate, allowing time for kidney transplant or regeneration.

A urinary tract infection (UTI) develops when part of the urinary tract becomes infected, usually by urease-producing bacteria. Urease breaks down urea, releasing ammonia into the systemic circulation. This may overwhelm the urea cycle, resulting in hyperammonaemia and coma (De Jonghe et al., 2002; Samtoy and Debeukelaer, 1980; Sato et al., 2008). Breath ammonia has the potential to serve as a diagnostic tool for UTIs and monitor treatment.

The effects of ammonia on the central nervous system

As has already been discussed, several dysfunctions of ammonia metabolism lead to hyperammonaemia and consequent HE and most of the impact of hyperammonaemic disease is on the CNS, predominantly the brain (Ong et al., 2003). Ammonia can cross the BBB to reach levels over 400 μM in the CNS, leading to neurological deterioration (Felipo and Butterworth, 2002; Munoz et al., 2000). Inherited defects such as UCDs and organic acidaemias are rare and are frequently undiagnosed at birth until significant and irreparable neurological impairment has occurred (Leonard and Morris, 2002; Prasad et al., 1997). It is not yet fully understood why the brain is more susceptible to permanent damage from elevated ammonia than other organs (Rose, 2014).

Ammonia is also a product of synthesis of glutamate from glutamine at nerve endings located in the CNS (Daniotti et al., 2011), (figure 4).

Glutamine is not toxic but it is osmotically active and accumulates, leading to astrocyte swelling, brain oedema, ICP and herniation (Albrecht and Norenberg, 2006). HE is a metabolic disorder which presents as a spectrum of neurological symptoms, principally as a consequence of hepatic dysfunction (Amodio et al., 2013; Cordoba et al., 1996; Cordoba and Minguez, 2008; Ferenci et al., 2002). The pathogenesis is not fully understood. Ammonia accumulation brought about by hepatic dysfunction and portosystemic shunting has been described as a primary cause. HE may be triggered by the intake of too much protein, dehydration, abnormal electrolyte homeostasis, gastrointestinal bleeding, infections and low blood oxygen. Elevated serum ammonia levels are detected in up to 80% of HE patients (Frontera, 2014). HE occurs in 30-45% of cirrhotic patients (Romero-Gomez et al., 2001) and 10-50% of those with transjugular intrahepatic portal-systemic shunts (Boyer and Haskal, 2010). The diagnosis is typically confirmed by blood ammonia determinations and electrophysical methods such as EEG (Eklou-Lawson et al., 2009). Psychometric tests can then be used to grade HE such as the Trail Making Test (TMT), West Haven Criteria (WHC), and the Glasgow Coma Scale (GCS). Newer techniques such as MRI, MRS and PET are then used to confirm diagnosis. Management of HE is broken into five steps; stabilisation, addressing modifiable factors, lowering blood ammonia, managing ICP and managing complications (Frontera, 2014). Drug treatment options include lactose and neomycin, along with a combination of rifraxamin and lactulose (Sharma et al., 2013). A liver transplant is considered to be a successful long-term therapy for HE. However, recipients who have HE at the time of a transplant are at high risk of neurological complications due to their susceptibility to stress of surgery and the neurotoxicity of drugs used in treatment (Dhar et al., 2008). As discussed, elevated blood ammonia levels are associated with kidney and liver dysfunction.

The digestive system

The digestive system converts food into energy. Food passes through the gastrointestinal tract which is made up of the oral cavity, pharynx, oesophagus, stomach, small and large intestines. The digestive system has long been acknowledged as a major source of ammonia. Ammonia is produced by protein breakdown and amino acid metabolism in the gastrointestinal tract. Bacteria in the gastrointestinal tract may also produce ammonia (Aprea et al., 2012).

The mouth contains a large and diverse microbial flora. As microbes accumulate, they form biofilms. Bacteria reside in these biofilms producing numerous VOCs. When bacterial levels are excessive due to poor oral hygiene, halitosis can result. This oral malodour is mostly due to by-products of microbial metabolism, principally sulphur and nitrogen compounds, including ammonia (Amano et al., 2002). Several studies have shown that oral bacteria contribute to ammonia levels measured in breath (Hibbard and Killard, 2011; Smith et al., 2008; Wang et al., 2008). In order to measure the correlation between blood ammonia and breath ammonia relating to physiological processes and not to bacteria in the mouth, antibacterial mouth rinses have been used (Solga et al., 2013). Nose exhalations may be used to measure ammonia levels instead of breath exhalations to avoid bacterial contributions (Barrow and Steinhagen, 1980). However, this is generally less acceptable for patients. Others have used sampling methods which exclude the oral breath fraction and capture only the late tidal volume to avoid this issue (Gouma, 2012). The contribution made by oral bacteria in the measurement of ammonia levels in breath sampling remains an issue of some debate in breath research (Schmidt et al., 2013).

H. pylori is a Gram negative spirillum bacillus, often found infecting the stomach and duodenum. Infection may be contracted from food or water. The bacteria can survive in the acidic environment of the stomach by secreting urease enzymes which generate ammonia to neutralise acids (Moblely et al., 1991). This weakens the lining tissue of the stomach causing ulcers. The ammonia produced may be released into systemic circulation causing hyperammonaemia. *H. pylori* infection is the main cause of peptic ulcer, chronic atrophic gastritis, gastric MALT lymphoma and gastric cancer. Elimination of *H. pylori* is performed in order to treat peptic ulcers, and is achieved using a combination of antibiotics (amoxicillin, clarithromycin, metronidazole) and a proton pump inhibitor (PPI) (lansoprazole and omeprazole), allowing the ulcer to heal naturally. One current method of diagnosis is the urea breath test (UBT) which involves measuring the urease activity of the organism via the ingestion of ^{13}C or ^{14}C -labelled urea. The generated labelled CO_2 then diffuses into the blood and to the lungs where it can be detected using a mass spectrometer (Cao and Duan, 2006). Stool samples, (Erzin et al., 2004) gastric juice (Mokuolu et al., 1997) and gastric biopsies (Cutler et al., 1995) may also be used to test for the presence of *H. pylori*. The application of ammonia-based breath tests without the need for radioactive labels is being explored (Kearney et al., 2002; Penault et al., 2005). However, no definitive clinical test based on breath ammonia is yet available and methodological issues remain, such as optimisation of the urea dosage, background ammonia production from *H. pylori* and metabolism of the generated ammonia by the liver.

In the intestines, the majority of ammonia production is due to digestive amino acid breakdown, predominantly glutamine (Turner et al., 2006). Significant levels of ammonia are also produced by bacterial breakdown of amino acids and urea (Damink et al., 2009). Amino acids, nucleotide

bases, and other nitrogenous compounds then diffuse into the blood and are transported to the liver (Berg et al., 2002). The highest ammonia concentration in the body is found in the colon. As ammonia is absorbed through the colonic epithelium, levels of L-glutamine and L-arginine in the portal blood are increased (Eklou-Lawson et al., 2009). Thus, there may be a metabolic link between colon mucosa and liver biosynthesis. Small intestinal bacterial overgrowth in patients with liver cirrhosis is more frequent in alcoholic liver cirrhosis cases.

Diet also affects ammonia metabolism and can be used as a tool to manage many disorders associated with hyperammonaemia. It has been found that ammonia emissions from the skin and blood concentrations increased after protein intake and reached maximum levels after two hours (Tsuda et al., 2011). In another study, volunteers fasted and then had a protein-rich meal. Breath ammonia concentrations fell immediately to one-half of fasting levels before an increase of two- or three-fold after five hours (Smith et al., 1999). The initial decrease may relate to increased clearance of ammonia by the liver, while the subsequent rise may be due to levels of nitrogen in the meal. Protein intolerances and related deficiencies (as mentioned in table 1) can prevent normal ammonia metabolism, causing severe and often irreversible damage (Sebastio et al., 2011; Shaw et al., 1989). Breath ammonia along with other metabolites has the potential to study gastrointestinal physiology (Spacek et al., 2015).

Glutamate dehydrogenase (GLDH) has been used as a marker of liver function as well as a marker for recent alcohol consumption in alcoholics (Kravos and Malesic, 2010). This reaction proceeds towards the direction of oxidative deamination of glutamate, which releases ammonia, normally with an activity of 6.4 U/L for women and 11.0 U/L for men. However, these values

are higher in alcoholics (Jung et al., 1985). GLDH and ammonia levels decrease rapidly after cessation of alcohol (Smith et al., 2002). There is the potential to replace the current GLDH test with a breath ammonia assay using orally administered glutamate, which could be used to monitor and screen for liver dysfunction and alcohol activity (Adeva et al., 2012). *H. pylori* infection is also common in alcoholics, resulting in the breakdown of urea to ammonia (Lieber, 1998). Mutations of glutamate dehydrogenase 1 (GDH1) can occur. This may cause HI/HA which is characterised by hypoglycaemic hyperinsulinaemia along with elevated blood ammonia, which requires monitoring and management.

The Lungs

Hyperventilation is an early sign of the metabolic crisis associated with hyperammonaemia, followed by encephalopathy. Hyperventilation can occur as a response to acidosis in order to improve carbon dioxide removal (Tizianello et al., 1977). Liver disease together with abnormal pH balance are likely to cause respiratory alkalosis. Altered consciousness along with respiratory alkalosis/acidosis should prompt the determination of blood ammonia (Krivitzky et al., 2009; Msall et al., 1984). Blood ammonia with transaminases should also be considered for perinatal asphyxia markers (Esque-Ruiz et al., 2003).

Asthma is a chronic inflammatory disease of the airways which affects about 10-25% children of the western world. It is usually diagnosed using respiratory function tests. Breath markers for the disease include nitric oxide and ammonia (Hunt et al., 2002). Although these markers have the potential to diagnose and monitor the disease, they may not replace simple respiratory function tests. Breath ammonia monitoring may also contribute to the management of cystic fibrosis

(Newport et al., 2009), although may not be likely to compete with the current means of diagnosis based on salt in sweat.

Ammonia is also added to tobacco to increase its alkalinity and therefore the proportion of free nicotine (van Amsterdam et al., 2011). Breath ammonia levels can be used to monitor cessation of smoking for upcoming surgery, or relevant quitting incentives, although CO is also considered a specific, if relatively insensitive marker of smoking activity (Bloor et al., 2008).

The pulmonary pathogen *Mycobacterium tuberculosis* is responsible for nearly two million deaths per year. Early diagnosis is vital to control the disease which spreads via exhalation. Ammonia, along with other VOCs could be considered for the diagnosis of tuberculosis (McNerney et al., 2012). The likelihood of VOC analysis being used to diagnose any inflammatory disease is low as these claims are not widely supported in the literature.

Muscle metabolism and exercise

Since the early 1920s it has been known that ammonia is released during skeletal muscle movement (Dawson, 1978). However, the importance of skeletal muscle in ammonia homeostasis was not recognised until the 1970s (Dawson, 1978). Abnormal nitrogen metabolism is caused by increased production of ammonia (seizure with increased movement of muscle) or impaired clearance of ammonia (kidney and liver dysfunction, portosystemic shunting) causing hyperammonaemia. The skeletal muscle becomes the most important organ in ammonia homeostasis during liver or kidney dysfunction (Lockwood et al., 1979). Skeletal muscle has a large mass capacity to remove ammonia by producing glutamine through the enzyme glutamine synthetase and through the purine nucleotide cycle (Sabina et al., 1984). At rest, there is no

uptake or release of ammonia by skeletal muscle. The intensity of exercise governs how ammonia is released into the venous blood of the exercising limb, either by the purine nucleotide cycle during brief exercise or by increased metabolism of branched chain amino acid breakdown (BCAA) during prolonged exercise (Derave et al., 1997; Lowenstein, 1990; Maclean et al., 1992).

Physical exercise has been used to study ammonia metabolism (Solga et al., 2014; Wilkinson et al., 2010). High intensity exercise induces exhaustive anaerobic metabolism as the body approaches hypoxia (Kato et al., 2004). This can cause an increase in lactate and ammonia in blood (Broomfield and Grunewald, 2012). During maximal exercise, ammonia and lactate levels decrease because of a reduction in blood pH caused by hypercapnia due to respiratory acidosis (Kato et al., 2005). Since ammonia is pH-dependent, breath testing can be linked to protein metabolism and changes of blood pH under exercise (Schubert et al., 2012). Ammonia produced during exercise has been shown to induce immune and inflammatory responses (Gleeson, 2007). To counter these responses, arginine supplements have been shown to decrease hyperammonaemia and lymphocyte response during intense exercise and the use of amino acids can modify metabolism during exercise (Goncalves et al., 2012). This increase of blood ammonia can be used to optimise exercise duration (Yges et al., 1999). Blood ammonia has been analysed before and after a handball game. Results demonstrated the inflammatory response effects lasted for 24 hours (Chatzinikolaou et al., 2014). The relationship between blood lactate, blood ammonia and heart rate can be assessed during exercise training to provide guidelines for athletic training schedules (Roeykens et al., 1998). In order to evaluate, monitor and prescribe exercise intensity for conditioning programmes, ammonia and lactate levels can be used

(Gorostiaga et al., 2010). Gender and age also need to be considered when compiling a conditioning programme (Lourenco and Turner, 2014).

Cardiovascular markers

There is ongoing work to find an ideal biomarker for the diagnosis of cardiac arrest. Blood ammonia was associated with poor neurological outcomes in a patient population being treated with therapeutic hypothermia (Cho et al., 2012). Pulmonary arterial hypertension is a progressive and devastating condition characterised by vascular smooth muscle and endothelial cell proliferation, leading to vascular narrowing that results in elevated pulmonary artery pressure, right ventricle failure and premature cell death (Badesch et al., 2009). Current biomarkers are invasive. Breath ammonia among other compounds has been seen to be elevated in patients, and ammonia correlated with the severity of the disease (Cikach et al., 2014). Non-invasive, continuous monitoring of breath ammonia could represent an effective, real-time means of monitoring cardiac arrest patient status.

Conclusions

Ammonia is involved in many processes in the body. As a consequence, it can be used to diagnose and monitor a number of conditions, either alone, or in combination with other tests and biomarker profiles. Breath ammonia analysis is still in its infancy. However, it is attracting increasing interest as a non-invasive means of diagnosis that has the potential to give convenience and clarity on the determination of systemic ammonia levels. While the use of breath ammonia holds great potential as a non-invasive diagnostic solution, many challenges remain, particularly establishing adequate evidence of the links between breath ammonia, blood

ammonia and disease status. Establishing such evidence has been held back by the quality of the available technology to perform such studies. New technology is required which has excellent analytical performance, which allows accurate measurement across the diagnostically relevant range and which is also able to perform large cohort studies through which such proofs can be determined. Application of suitable technology, followed by clinical evidencing has the potential to lead to a broad range of screening, monitoring and diagnostic solutions for the conditions discussed.

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Table 1. Pathological conditions associated with hyperammonaemia.

Classification	Underlying causes	Ammonia (μM)	Details	Treatment
Inherited	Transient hyperammonaemia of new-borns	>1500		Lactitol, carglumic acid
	Urea cycle disorders	>600	Enzyme and transporter defects, citrin deficiency, hyperornithinaemia, hyperammonaemia, homocitrullinuria, lysinuric protein intolerance	Sodium benzoate, branched chain amino acids (BCAAs), glycerol phenylbutyrate. Liver (Mukhtar et al., 2013) and/or kidney (Bezinover et al., 2010) transplant
	Organic acidaemias	100-150~600	Pyruvate dehydrogenase deficiency, Type B pyruvate carboxylase deficiency, Propionic, methylmalonic, isovaleric	Biotin, thiamine, dichloroacetate, citrate
	Respiratory alkalosis	~200	Hepatic glutamine synthetase deficiency, primary pulmonary hypertension and high nitrogen load	Lung transplant (Hocker et al., 2011; Lichtenstein et al., 2000)
	Fatty acid oxidation disorders	200-600	Carnitine palmitoyltransferase-1/ long chain 3-hydroxyacyl-CoA dehydrogenase deficiency/ very long chain hydroxyacyl-CoA dehydrogenase deficiency/ glutaric aciduria type II/ carnitine deficiency	Bone marrow transplant (Davies et al., 1996)
	Hyperinsulinism hyperammonaemia	~250	Hypoglycaemia and	Diazoxide, K_{ATP} antagonist,

	syndrome		hyperinsulinaemia	epigallocatechin gallate
Acquired	Sepsis, liver dysfunction	<200		BCAAs, L-orthinine, L-aspartate, glycerol phenylbutyrate

Table 2. Urea cycle disorders.

Enzyme	Role and deficiency	Symptoms
N-acetylglutamate synthase (NAGS)	Catalyses synthesis of NAG from acetyl-CoA-1 and glutamate which activates CPS-1	Congenital: respiratory alkylolosis, hyperammonaemia, coma
		Acquired: Acute attacks of hyperammonaemia, neurological, gastrointestinal and psychiatric clinical signs. it may be developed secondary to carnitine deficiency
Carbamoylphosphate synthetase-1 (CPS-1)	Catalyses the first step of the urea cycle; synthesis of carbamoyl phosphate from HCO_3^- , ATP, and NH_3 using NAGS	Congenital: hyperammonaemia, coma, delayed development, intellectual disability
		Acquired: secondary to HI/HA syndrome
	Urea cycle cannot proceed without carbamoylphosphate	
Ornithine transcarbamylase (OTC)	Catalyses the synthesis of citrulline from carbamoylphosphate and ornithine that enters the mitochondria from the cytosol	Congenital: hyperammonaemia, respiratory alkalosis and cerebral oedema
		Acquired: triggered by catabolism
Argininosuccinate synthetase-1	Combines citrulline and aspartate in the cytosol generating argininosuccinate	Type 1 citrullinemia, cancer
Argininosuccinate lyase	Catalyses the breakdown of argininosuccinate to arginine, arginase and fumarate	Argininosuccinic aciduria, HE, respiratory alkalosis with neurological manifestations, reduced arginine synthesis
Arginase-1	Catalyses the hydrolysis of arginine to ornithine and urea	May go undiagnosed until later in life and recognised as cerebral palsy, spastic tetraplegia in children

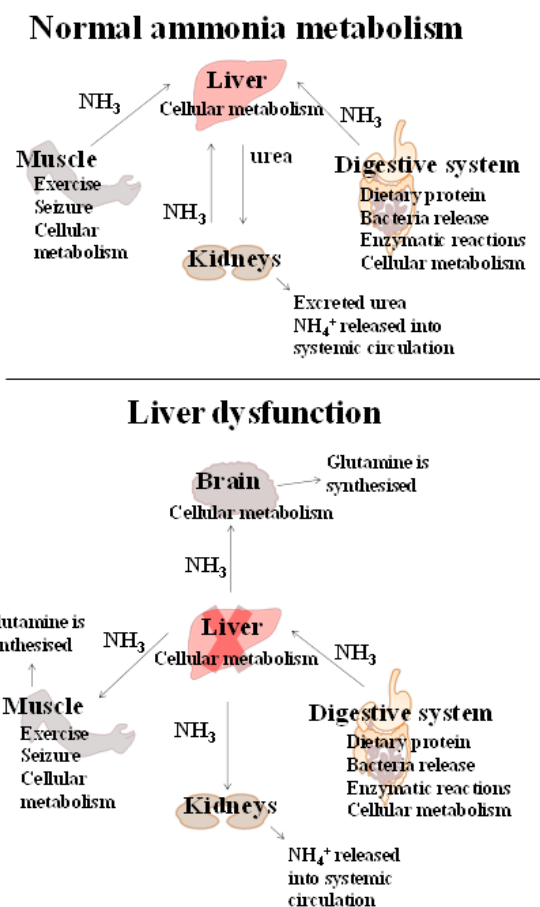


Figure 1 Schematic of ammonia metabolism under normal conditions and during liver dysfunction. Endogenous or exogenous ammonia is transferred to the liver and detoxified. It is then transferred to the kidneys and excreted as urea. During liver dysfunction ammonia is not detoxified and accumulates in the body, passing through the blood-brain barrier. Skeletal muscle also begins to use up excess ammonia, generating glutamine.

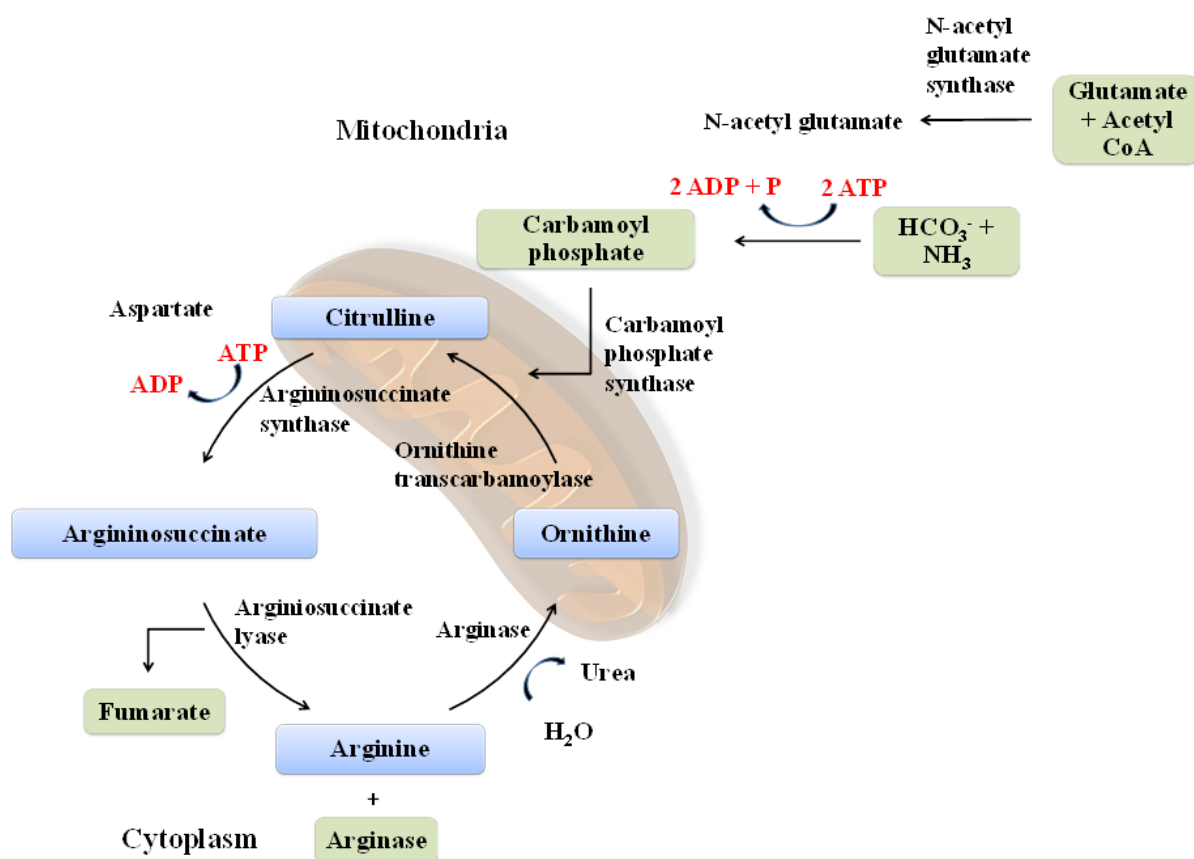


Figure 2 The urea cycle. N-acetylglutamate (NAG) is synthesised from glutamate and acetyl-CoA by N-acetylglutamate synthase (NAGS). This activates carbamoyl phosphate synthetase-1 (CPS-1) to initiate the urea cycle. Ammonia is first absorbed into the liver and combined with bicarbonate to form carbamoyl phosphate in the mitochondrial matrix. This enters the urea cycle and combines with ornithine (from the cytoplasm) to form citrulline. In the cytosol, amino acids are fed into the cycle by aspartate which combines with citrulline to form argininosuccinate. Argininosuccinate is then split into fumarate (which is fed into the citric acid cycle) and arginine. Arginine then reacts with arginase and water to produce urea and regenerated ornithine. This travels from the mitochondrial matrix via the ornithine transporter, so completing the cycle.

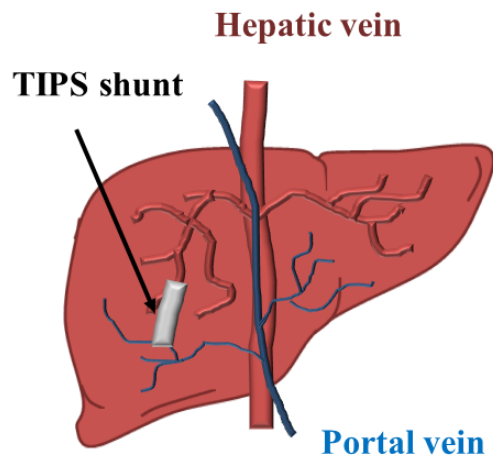


Figure 3 Blood from the gastrointestinal tract and spleen is carried by the portal vein and diverted to the hepatic vein, bypassing the liver via a portosystemic shunt.

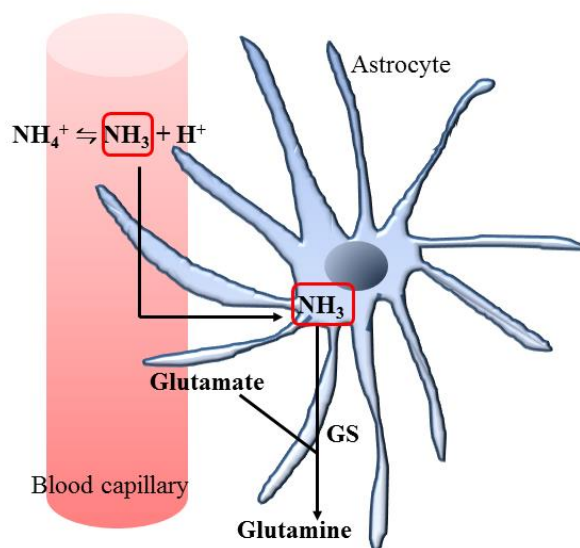


Figure 4 Ammonia can pass through the BBB and into brain astrocytes. Here, ammonia may be metabolized to glutamine via glutamine synthetase (GS).